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Insulin Promotes Diacylglycerol Kinase Activation by Different Mechanisms in Rat Cerebral Cortex Synaptosomes

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The mechanism by which insulin increases diacylglycerol kinase (DAGK) activity has been studied in cerebral cortex (CC) synaptosomes from adult (3–4 months of age) rats. The purpose of this study was to identify the role of phospholipases C and D (PLC and PLD) in DAGK activation by insulin. Neomycin, an inhibitor of PLC phosphatidylinositol-bisphosphate (PIP_2) specific; ethanol, an inhibitor of phosphatidic acid (PA) formation by the promotion of a transphosphatidyl reaction of phosphatidylcholine phospholipase D (PC-PLD); and DL propranolol, an inhibitor of phosphatidate phosphohydrolase (PAP), were used in this study. Insulin (0.1 μM) shielded an increase in PA synthesis by [^{32}P] incorporation using [γ-^{32}P]ATP as substrate and endogenous diacylglycerol (DAG) as co-substrate. This activated synthesis was strongly inhibited either by ethanol or DL propranolol. Pulse chase experiments also showed a PIP_2-PLC activation within 1 min exposure to insulin. When exogenous unsaturated 18:0-20:4 DAG was present, insulin increased PA synthesis significantly. However, this stimulatory effect was not observed in the presence of exogenous saturated (di-16:0). In the presence of R59022, a selective DAGK inhibitor, insulin exerted no stimulatory effect on [^{32}P]PA formation, suggesting a strong relationship between increased PA formation by insulin and DAGK activity. These data indicate that the increased synthesis of PA by insulin could be mediated by the activation of both a PC-PLD pathway to provide DAG and a direct DAGK activation that is associated to the use of 18:0-20:4 DAG species. PIP_2-PLC activation may contribute at least partly to the insulin effect on DAGK activity.

Key words: phosphatidic acid; diacylglycerol; insulin; synaptosomes

Insulin (I) and insulin receptors (IRs) are both present in the brain. Insulin is actively transported across the blood–brain barrier and it seems to be locally produced in the brain (Unger et al., 1991; Schulingkamp et al., 2000). The insulin receptor (IR) has been expressed widely in the central nervous system (CNS) (Baskin et al., 1988; LeRoith et al., 1988; Unger et al., 1991). It has also been suggested that in this tissue IR-mediated functions regulate cognition and memory processes (Zhao et al., 2001). Insulin receptors are located at the synapse where they regulate neurotransmitter release and receptor recruitment, indicating a potential involvement of insulin in synaptic plasticity (Abbott et al., 1999).

We have reported recently that insulin induces an increase in PA synthesis in CC synaptosomes through the activation of DAGK activity (Salvador et al., 2005). This study reported that insulin increases PLD activity whereas PAP2 activity remains unchanged. This observation resulted from the use of specific radiolabeled substrates for the determination of DAGK, PLD, and PAP2 activities and similar conditions for the evaluation of insulin action.

The role of DAG and PA as lipid second messengers have become the focus of attention of several studies. DAGK activity that removes DAG attenuating PKC signaling and, in doing this, yields a highly active metabolite (i.e., PA), seems to participate in modulating the balance between these bioactive lipids.

Phosphatidic acid is an intracellular phospholipid second messenger whose production is stimulated by an array of ligands. Phosphatidic acid mediates a variety of biologic effects in the absence of a conversion mechanism to other second messengers such as DAG. Exogenous PA induces DNA synthesis, myc/fos expression, and hormone secretion (Yu et al., 1988; Van Corven et al., 1992) as well as the modulation of actin polymerization (Lee et al., 2003).

Cellular targets for PA include PI-4-kinase, PKC ξ, MAP kinase, protein tyrosine phosphatases, and raf-1

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(Zhao et al., 1993; Jenkins et al., 1994; Limatola et al., 1994; Siddiqui and Yang, 1995; Ghosh et al., 1996). The potency of PA in cell systems generally induces a more prominent effect for molecular species containing longer fatty acids with higher degrees of unsaturation (Krabak and Hui, 1991; Pearce et al., 1994).

It is known that PA production from membrane phospholipids may be mediated by: 1) the activity of PLC acting on either PIP_2 or PC, and sequentially by the action of DAGK activity; and 2) PLD activity on PC. PLD/PAP2 pathway activation induces PA metabolization with DAG generation and DAGK could be sequentially activated. The activation of DAGK therefore occurs partially via the production of DAG at the plasma membrane, acting as a substrate for the kinase.

It has been reported recently that ligand stimulation of epidermal growth factor receptor (EGFR) in NIH3T3 cells stimulates PA production through DAGK activation by a PLD-independent mechanism (Montgomery et al., 1997). In other cells, however, the stimulation of EGFR increases PA production either through one or both of these pathways (Payrastre el al., 1991; Kaszkin et al., 1992; Song et al., 1994). In quiescent Swiss 3T3 cells stimulated with a mitogenic concentration of IGF-I, DAGK activation has been reported to be involved in signal deactivation (Divecha et al., 1991).

In the present study we have examined insulin action on synaptosomal PA generation by DAGK activity when PIP_2-PLC and PLD/PAP2 specific inhibitors are present.

To ascertain either whether or not an increase in DAG levels is sufficient by itself to induce an increase in DAGK activity, or whether or not IR stimulation is required for its activation: 1) DAG levels were increased adding either di-16:0 DAG or 18:0-20:4 DAG and DAGK activity was measured with [γ-^{32}P]ATP; and 2) DAGK activity was measured with exogenous added [^{3}H]DAG as radioactive substrate. In both cases, DAGK activity was determined in either the absence or the presence of insulin.

We report that in CC synaptosomes, insulin stimulates PA synthesis through a DAGK activity in two different ways: 1) through the use of DAG produced either by PIP_2-PLC or by PLD-PAP2 activated pathways; and 2) by DAGK activation, independently of membrane DAG generation that selectively promotes PA synthesis from 18:0-20:4 DAG species.

MATERIALS AND METHODS

Wistar-strain rats were kept under constant environmental conditions and fed on a standard pellet diet. [2-^{3}H]Glycerol (200 mCi/mmol), [γ-^{32}P]ATP (3,000 Ci/mmol), and Omnifluor were obtained from New England Nuclear-Dupont (Boston, MA). All the other chemicals were from Sigma-Aldrich (St. Louis, MO).

Preparation of Subcellular Fractions

Total homogenates were prepared from the cerebral cortex (CC) of 4-month-old rats. Rats were killed by decapitation and CC was immediately dissected (2–4 min after decapitation).

Synaptosomal fraction was obtained as described previously by Cotman (1974) with slight modifications (Salvador et al., 2002). Briefly, CC homogenate was prepared in a buffer containing 20% (wt/vol) in 0.32 M sucrose, 1 mM EDTA, 10 mM buffer HEPES (pH 7.4) in the presence of 1 mM DTT, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 0.1 mM PMSF.

The CC homogenate was centrifuged at 9,600 × *g* for 7.5 min using a JA-21 rotor in a Beckman J2-21 centrifuge and the supernatant was carefully poured into another tube. The supernatant was subsequently centrifuged at 26,400 × *g* for 20 min to obtain the crude mitochondrial pellet (CM). The CM was washed and resuspended with 3 ml of the isolation medium and loaded onto a 8.5% Ficoll medium (6 ml) and 13% Ficoll medium (6 ml) discontinuous gradient. The Ficoll solutions were prepared in the isolation medium. The sample loaded onto the discontinuous gradient was centrifuged at 134,000 × *g* for 30 min using a SW 28.1 rotor in a Beckman L5-50 ultracentrifuge. The myelin fraction band was at the interface between the isolation medium and the 8.5% Ficoll medium, the synaptosomal band at the interface between the 8.5% and the 13% Ficoll media, and the free mitochondrial fraction was the pellet below the 13% Ficoll medium. Synaptosomal fraction was centrifuged at 40,800 × *g* for 20 min using a JA-21 rotor in a Beckman J2-21 centrifuge and resuspended in the assay buffer.

Determination of DAGK Activity

DAGK activity was determined in the synaptosomal membranes either by measuring radioactive phosphate incorporation into PA using [γ-^{32}P]ATP and endogenous DAG as substrates, [γ-^{32}P]ATP and exogenous DAGs (di16:0 DAG or 18:0-20:4 DAG), or [^{3}H]DAG as radioactive substrate and 1 mM ATP. The standard assay contained in a volume of 200 μl, 50 mM HEPES, pH 7.4, 20 mM NaF, 10 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP. When radioactive or free DAG was added to the assay, it was resuspended in 1% DMSO and detergent was at a final assay concentration of 0.1%. To compare all experimental assays, DMSO (0.1%) was also present when [γ-^{32}P]ATP and endogenous DAG were employed. Reactions were carried out at 37°C at 2 and 5 min. Reactions were stopped by adding chloroform/methanol/1N HCl (2:1:0.2, by vol) and lipids were extracted by Folch et al. (1957). Blanks were prepared identically except that membrane fractions were boiled for 5 min before being used. Lipid products synthesized from [^{3}H]DAG as precursor were separated by TLC on silica gel G (Giusto and Bazán, 1979) and developed with hexane/diethyl ether/acetic acid (30:70:1, by vol). For [^{3}H]MAG isolation, the plate was rechromatographed up to the middle by using hexane/diethyl ether/acetic acid (20:80:2.3, by vol) as developing solvent. In this experimental approach, [^{3}H]Glycerol was measured as a product of monoacylglycerol lipase activity in the methanol:aquous layer of the Folch procedure.

When [γ-^{32}P]ATP was employed as radioactive substrate, reactions were also stopped by adding chloroform/methanol/

1N HCl (2:1:0.2, by vol) and lipids were extracted by Folch et al. (1957). Lipid extracts were washed five times with theoretical upper phase to eliminate [γ-^{32}P]ATP. Phosphatidic acid was separated by TLC on 1% potassium oxalate in silica gel H and developed with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7.5, by vol). Lipids were visualized by exposure of the chromatograms to iodine vapors and scraped off to a vial for counting by liquid scintillation after the addition of 0.4 ml water and 10 ml 5% Omnifluor in toluene:Triton X-100 (4:1 by vol).

Determination of PIP_2-PLC Activity

Purified synaptosomal membranes were resuspended in the DAGK buffer assay and sonicated in the presence of 10 μM [γ-^{32}P]ATP (16 μCi/mg of synaptosomal protein). Pulse-label was carried out at 37°C during 10 min and stopped by the addition of a precursor-free medium to a final ATP concentration of 3 mM. Immediately after removing zero-time samples, the suspension was re-incubated (chase period) during 1, 4, 5, and 6 min and it was stopped subsequently by the addition of chloroform/methanol/1N HCl (2:1:0.2, by vol). Lipids were extracted as described previously. PIP_2 was separated by TLC on 1% potassium oxalate in silica gel H and developed with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7.5, by vol). The position of PIP_2 was visualized by iodine exposure and identified by using PIP_2 standard. Silica gel spots were scraped off to a vial for counting by liquid scintillation spectroscopy as described previously.

Preparation of Radioactive 1,2-Diacyl-Sn-Glycerol

Radioactive DAG was obtained from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol) as described previously (Pasquaré de García and Giusto, 1986). Lipids were extracted from the tissue as described in Folch et al. (1957). [2-^{3}H]DAG plus cholesterol were isolated by mono-dimensional TLC in silica gel G plates and with a solvent system of hexane:ether:acetic acid (60:40:2,3 by vol). Lipids were eluted (Arvidson, 1968) and DAG was purified by one-dimensional TLC on silica gel G developed with chloroform:methanol:-acetic acid (98:2:1, by vol). The substrate was eluted as above from the silica gel and stored in chloroform solution to avoid the production of 1,3-diacyl-sn-glycerol.

Other Methods

Protein and lipid phosphorus were determined according to Bradford (1976) and Rouser et al. (1970), respectively.

Statistical Analysis

Statistical analysis was carried out using Student's *t*-test with the values representing the mean ± SD of six individual samples per condition. Each experimental data was obtained from two different experiments. Each experiment was carried out using three rats. Synaptosomes from six rats were then assayed. In ratios, the SD was calculated according to the ad hoc statistical treatment (Johnson and Kotz, 1969).

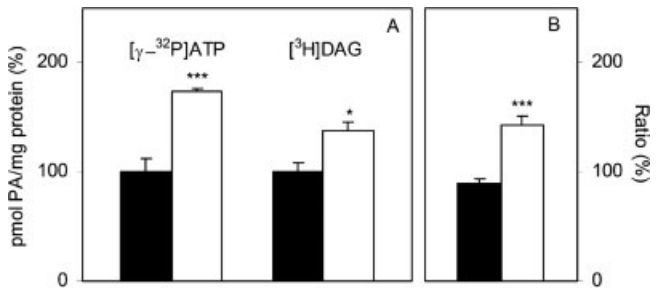

Fig. 1. Insulin effect on synaptosomal DAGK activity measured with either endogenous or exogenous substrate. **A:** DAGK activity was determined in CC synaptosomal membranes either measuring radioactive phosphate incorporation into PA through [γ-^{32}P]ATP as radioactive substrate and synaptosomal DAG as lipid substrate or measuring PA synthesized from [^{3}H]DAG as radioactive substrate and 1 mM ATP when insulin 0.1 μM (□) or vehicle (■) was present. Incubation time with radioactive substrate = 5 min. **B:** Represented the ratio ×100 between PA and MAG + glycerol using [^{3}H]DAG and ATP 1 mM as substrate. The lipid was resuspended in DMSO at a final concentration of 0.1% in the assay condition as described under Materials and Methods. Results are calculated as percentages of incorporation values (5-min incubation period). Controls are set at 100 and the values are means ± SD of six individual samples. $*P < 0.050$, $**P < 0.010$, $***P < 0.001$ (significance degree were insulin vs. control).

RESULTS

Insulin Promotes an Increase in PA Generation Through the Activation of DAGK Activity

DAG kinase is an enzyme that attenuates the level of DAG generated in the membrane by converting DAG into PA. To examine the mechanism of insulin action, CC synaptosomes were incubated in the presence of different radioactive precursors and the hormone. In the presence of [γ-^{32}P]ATP, DAGK activity was measured using synaptosomal endogenous DAG. This type of assay depends on the availability of DAG in the system because DAG generated by different enzymatic pathways (PLD/PAP2, PC-PLC, PIP_2-PLC) could be used as DAGK substrate. When radioactive DAG was used as substrate, a micellar assay was carried out with DMSO. In this DAGK assay, PA generation was independent of the endogenous DAG levels. A concentration of 0.1% DMSO was used in both enzymatic assays.

As shown in Figure 1A, the presence of 0.1 μM insulin increases PA formation through [γ-^{32}P]ATP and this phospholipid represents 73% over the control value. Under these conditions, the content of PA synthesized in the absence of insulin was 12.21 ± 1.51 pmol/mg protein. [^{3}H]PA synthesized through [^{3}H]DAG is also represented in Figure 1A. In this experimental approach, lipid substrate (DAG with a complex fatty acid composition) was present at 250 μM to minimize dilution with endogenous DAG. In the presence of insulin, DAGK activity evidenced a 46% increase with respect to controls. Under these conditions, monoacylglycerol (MAG)

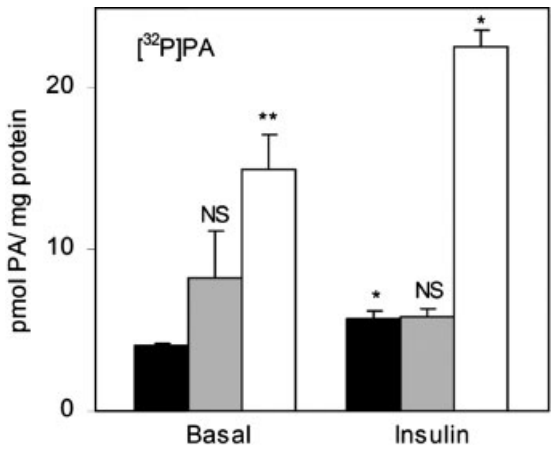

Fig. 2. Insulin effect on synaptosomal DAGK activity measured with [γ-^{32}P]ATP and either saturated or unsaturated DAG. The enzymatic assay was conducted during 5 min with [γ-^{32}P]ATP (0.1 mM) as radioactive substrate either in the absence (black) or presence of 50 nmol/sample of di-16:0 (gray) or 18:0-20:4 diacylglycerol (white). The lipidic substrate was resuspended in DMSO at a final concentration of 0.1% under the assay conditions described in Materials and Methods. Incubation time with radioactive substrate = 5 min. Results are expressed as the mean ± SD of the values obtained in six individual samples. *$P < 0.050$, **$P < 0.010$, ***$P < 0.001$. In basal conditions, the significance degrees were dipalmitin vs. control and 18:0-20:4 vs. control. Under insulin conditions, the significance degrees were calculated vs. its controls without insulin.

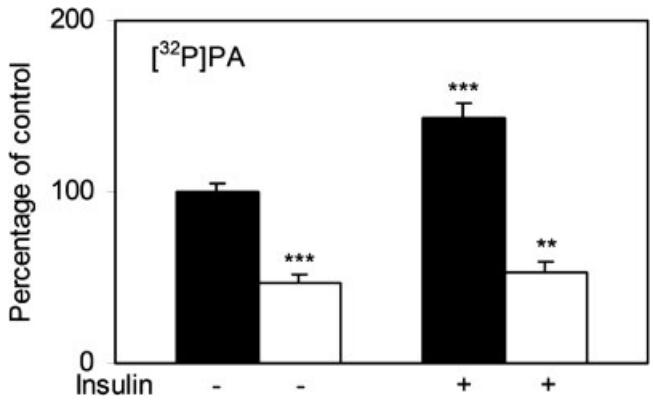

Fig. 3. DAGK involvement in insulin action. R59022 effect. **A:** DAGK activity was determined in CC synaptosomal membranes after 5 min of incubation by measuring [^{32}P]PA in either the absence or presence of insulin when vehicle (■) or R59022 10 μM (□) was present. Results are calculated as percentages of incorporation values. Controls are set at 100 and the values are means ± SD of six individual samples. *$P < 0.050$, **$P < 0.010$, ***$P < 0.001$. The significance degrees were calculated vs. control without insulin and R59022.

and glycerol production were also evaluated representing 32% and 21%, respectively. Results obtained with PA, MAG, and soluble products were expressed as a ratio between the kinase activity (DAGK) and the hydrolytic activity (MAG + Glycerol). As shown in Figure 1B, insulin exhibited an increase in the use of DAG and the stimulatory effect on the kinase activity was over the hydrolytic activity.

To explore insulin effect (independent of DAG generation) with [γ-^{32}P]ATP), 250 μM of exogenous DAG was employed in the DAG-detergent micellar assay. Under these conditions, DAGs with different fatty acid composition could be evaluated. It is known that one of nine DAGK isoforms is selective with respect to the DAG fatty acid composition. Only DAGKε shows selectivity to 18:0-20:4 species of DAG (Luo et al., 2004). In our experimental approach, dipalmitin and 18:0-20:4 DAG were used as non-labeled co-substrates.

As shown in Figure 2, the exogenous substrate increased PA formation with respect to that obtained with endogenous synaptosomal DAG content. Whereas 250 μM dipalmitin showed a tendency to increase PA formation, 18:0-20:4 DAG increased both strongly and significantly PA synthesis (300% with respect to control). Figure 2 also shows the effect of insulin when 250 μM DAGs are present. In the presence of dipalmitin, insulin induced no increase PA synthesis with respect to its control without the hormone. However, in the presence of 18:0-20:4 DAG, insulin showed a strong and significant stimulatory effect with respect to its control ($P < 0.005$).

These results indicate that insulin stimulates PA formation by means of two mechanisms. One of them seems to be independent of substrate concentration. It is possible that the greatest effect evidenced by the hormone when [γ-^{32}P]ATP was used as radioactive substrate (73% over the control) could be due to the additional stimulation in DAGK activity by an increased DAG generation through phospholipase activities.

It is known that insulin increases de novo glycerolipid pathway in several tissues. In experiments with CC synaptosomal membranes and [2-^{3}H]Glycerol, as a selective precursor for glycerolipid synthesis, glycerophospholipid labeling was significant at 60, 90, and 120 min incubation (unpublished observations). PA, ^{32}P labeled using [γ-^{32}P]ATP) as substrate, could therefore be found. It was determined in these preparations of rat cortical synaptosomes (Pasquaré et al., 1986) that at short incubation times (5–15 min), PAP type 2 (PAP2) or NEM-insensitive activity, was predominant whereas PAP type 1 (PAP1) or NEM-sensitive activity, usually related to glycerolipid synthesis was not detected. These experiments were carried out with appropriate substrates and under appropriate conditions for these two activities.

To confirm the involvement of DAGK in this insulin effect, R59022, a potent and selective DAGK inhibitor (Jiang et al, 2000), was used. When it was present in the DAGK assay, PA formation from [γ-^{32}P]ATP was strongly reduced (Fig. 3). Under these conditions, insulin exerted no stimulatory effect on [^{32}P]PA formation. This suggests a strong relationship between increased PA formation by insulin and DAGK activity.

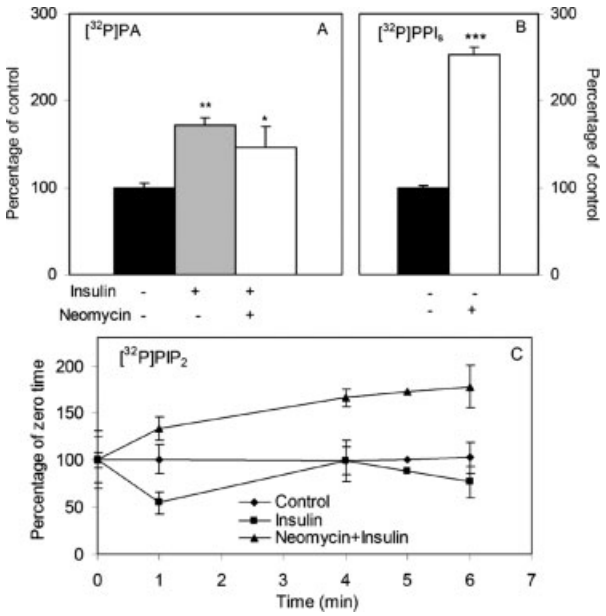

Fig. 4. Insulin effect on synaptosomal DAGK activity in either the absence or presence of neomycin. **A:** DAGK activity was determined in CC synaptosomal membranes after 2 min of incubation by measuring [^{32}P]PA when neomycin 0.1 mM or vehicle was present. Pre-incubation time with neomycin or vehicle = 10 min. **B:** Control of neomycin inhibition by measuring polyphosphoinositides. Results are calculated as percentages of incorporation values. Controls are set at 100 and the values are means ± SD of six individual samples. **C:** Pulse chase assay. Synaptosomes were incubated during 10 min in either the presence or absence of 0.1 mM of neomycin and 3.6 μCi per condition of [γ-^{32}P]ATP (pulse). The reaction mixture was subsequently re-incubated with 3 mM ATP by 1, 4, 5, and 6 min in either the presence or absence of insulin (chase). Results are calculated as percentages of incorporation values at zero time. Controls are set at 100 and values are means ± SD of six individual samples. *$P < 0.050$, **$P < 0.010$, ***$P < 0.001$.

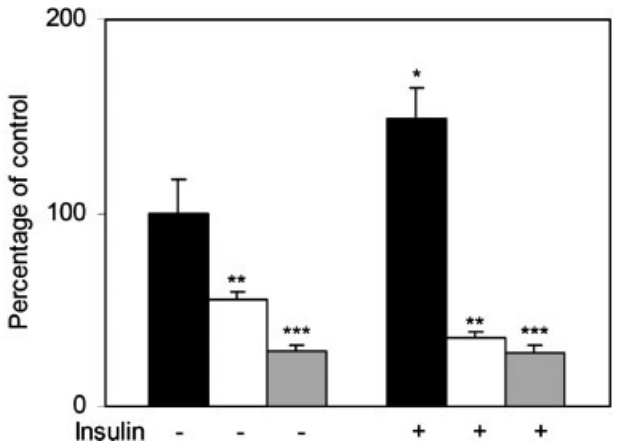

Fig. 5. Insulin effect on synaptosomal DAGK activity in either the absence or presence of ethanol and DL propranolol. DAGK activity was determined in CC synaptosomal membranes after 5 min of incubation by measuring [^{32}P]PA in either the absence or presence of insulin when vehicle (black), 2% ethanol (white), or 1.5 mM DL propranolol (gray) was present. Pre-incubation time with ethanol, DL propranolol, or vehicle = 10 min. Results are calculated as percentages of incorporation values. Controls are set at 100 and values are means ± SD of six individual samples. *$P < 0.050$, **$P < 0.010$, ***$P < 0.001$.

Is DAGK Activity in Insulin-Treated Synaptosomes Potentiated by an Increased DAG Generation?

We have reported recently that insulin increases PLD activity whereas PAP2 activity remains unchanged. This observation resulted from the use of specific radiolabeled substrates for the determination of these enzymatic activities and similar conditions for the evaluation of insulin action (Salvador et al., 2005). It can be suggested that PLD activation by insulin and subsequent PAP2 activity over PA is a potentially increased DAG generation mechanism.

DAG kinase is an enzyme that attenuates the level of DAG generated in the membrane by its conversion to PA. It has been reported that DAG is produced from several different sources in a biphasic manner in some, but not all, cells on stimulation. An initial phase, which appears within 1 min, is generated from PIP_2 by the action of PLC, and a second sustained phase is mainly generated from PC by the sequential action of PLD and PAP (Reibman et al., 1988; Fu et al., 1989).

To study the additional DAGK activation by the endogenous generation of DAGs, either PIP_2-PLC or PLD were explored as putative activated pathways.

DAGK activity was measured through ^{32}P incorporation into PA, using synaptosomal endogenous DAG generated by different phospholipases activities. Neomycin, a PIP_2-PLC inhibitor, was used to explore DAGK activation by insulin.

Figure 4A shows results from synaptosomal PA synthesis during 2 min incubation with [γ-^{32}P]ATP after a previous exposure (10 min) of membranes to neomycin. Insulin, added with radioactive substrate, exerted its stimulatory action in the presence of neomycin. Figure 4B shows the accumulation of radioactive polyphosphoinositides (PIPs) as a positive control of the inhibitory PIP_2-PLC action of neomycin.

To directly evaluate the insulin effect on PIP_2-PLC activity a pulse chase experiment was carried out.

A 10-min incubation of synaptosomes with [γ-^{32}P] ATP with and without neomycin was carried out. Samples were finally re-incubated with 3 mM ATP during 1, 4, 5, and 6 min, either in the absence or presence of insulin. Under these re-incubation conditions (chase period), the radioactive substrate was diluted (1:300).

As shown in Figure 4C, under control conditions no change in PIP_2 label was observed in the chase period. When insulin was present, however, a 44% loss of label with respect to zero time was observed at 1 min incubation. In the presence of neomycin, insulin failed to exert this effect. These data indicate that PIP_2-PLC was activated by insulin at short incubation times. In addition, after 5 min of incubation with the hormone, a

PIP_2 resynthesis took place. At this stage, a PIP_2 accumulation was observed in the presence of neomycin.

There was no increase in the labeling of phosphatidylinositol 4-phosphate (PI4P) or phosphatidylinositol (PI), whereas an increase of inositol phosphates was observed in the presence of lithium chloride (data not shown). It therefore seems unlikely that PIP_2 disappears as a result of phosphomonoesterase activation. Our results suggest that PIP_2 label was decreased by PLC activation. This was also indicated by the accumulation of PIP_2 when neomycin, a PIP_2-PLC inhibitor, was present in the medium with [γ-^{32}P]ATP (Fig. 4B).

To evaluate PC-PLD pathway participation in insulin-induced DAGK activation, 2% ethanol was used. Primary alcohols promote transphosphatidyl reaction of PC-PLD activity. This pathway and its influence on DAGK stimulation by insulin could thus be evaluated. As shown in Figure 5, insulin exerted no stimulatory effect in the presence of ethanol at 5 min incubation, and the resulting values were similar to those of controls with ethanol. This experiment was also carried out at 2 min incubation yielding a similar result (data not shown). It is worthy of note that in the presence of ethanol PA formation was 50% inhibited with respect to controls, indicating that PLD activity could be upstream of DAGK activity in CC synaptosomes.

Using DL propranolol, at the concentration reported to be inhibitory for all PAP type 2 isoforms (Sciorra and Morris, 1999), a strong inhibition in basal DAGK activity was observed and PA labeling represented only a 30% of control (Fig. 5). Taking into account that PA formation was 50% inhibited by ethanol in controls, it could be hypothesized that DAGs from other phosphatidate phosphohydrolase activity different from PAP2 that is PC-PLD associated, could also be a DAGK substrate. As shown in Figure 5, in the presence of the drug, no DAGK activation by insulin was observed.

To determine if DL propranolol exerts a direct inhibitory action on DAGK, another experiment was conducted. [^{3}H]DAG was used as the radioactive substrate. In the presence of 1.5 mM DL propranolol, [^{3}H]PA synthesis was similar to that under control conditions. In addition, DL propranolol did not affect insulin effect on DAGK when [^{3}H]DAG was the substrate (data not shown).

Results with ethanol and DL propranolol are indicative of PLD activation by insulin.

DISCUSSION

The present study shows that, after insulin action, PA formation is stimulated in CC synaptosomes. Phospholipase inhibitors were used to identify the role of two phospholipases in DAGK activation by insulin. Our results indicate that DAGK acts in response to increased DAGs generated by PIP_2-PLC and PLD-PAP2 pathways.

In the experiments with neomycin, used as PIP_2-PLC inhibitor, insulin seemed not to activate PIP_2 hydrolysis. However, data from pulse chase experiments show that insulin activates PIP_2 hydrolysis. The absence of PA formation in the presence of neomycin could be indicative of the following: 1) although PIP_2-PLC is inhibited partly by neomycin, an increased PIP_2 pool (Fig. 4B) led to an increase in DAGs resulting from this phospholipase activity; 2) a direct specific stimulatory effect of insulin on DAGK (as shown in Fig. 4) preferentially phosphorylates 18:0-20:4-DAG species; and 3) the contribution of DAG produced from PIP_2-PLC pathway to PA formation at 2 min is relatively small.

PIP_2-PLC activation by insulin in pulse-chase experiment was observed at short times of exposure (1 min) whereas data from inhibitors were obtained at 2 and 5 min of incubation. It is possible that after 1 min of exposure to the hormone, DAG is mainly generated by PLD-PAP2 pathway.

As shown in Figure 5, DAGK activation by insulin (5 min) was completely abolished in the presence of 2% ethanol. Ethanol, a PLD transphosphatidylating activator, stimulated phosphatidylethanol (Peth) synthesis. These data indicate that DAGK is a downstream effector in PLD activation by insulin.

These data are in accordance with a previous study from our laboratory in which radioactive PC was used as substrate and the PC-PLD pathway from CC synaptosomes was stimulated by insulin at short incubation times (Salvador et al., 2005).

We have reported recently the co-existence of PC-PLC and PC-PLD activities in rat cortical synaptosomes (Mateos et al., 2006). We have observed that DAGs from either PC-PLD or PC-PLC represent 25% and 75% of the total DAGs from PC hydrolysis. In these studies, we used 1-palmitoyl-2[^{14}C]arachidonoyl-sn-glycero-3-phosphocholine and 2% ethanol ([^{14}C]Peth, as the product of PLD transphosphatidylating activity) or D609, a PC-PLD inhibitor ([^{14}C]DAG as the product of PC-PLC activity). We found that in rat cortical synaptosomes the presence of 2% ethanol eliminates DAGs from PLD-PAP2 activities.

In addition, DAGK activation by insulin was completely abolished in the presence of 1,5 mM DL propranolol (Fig. 5). A direct inhibitory effect of DL propranolol on DAGK activity either in the presence or absence of insulin was discarded (data not shown).

These data indicate that insulin increased PLD activity and that PA produced by this pathway was metabolized by PAP2 to DAG. As reported previously, PAP2 activity seems not to be modified by insulin (Salvador et al., 2005). In this study, 0.5 mM of labeled PA was reported to be used as substrate. Therefore, an increase in PA formation from PLD activity in the absence of exogenous PA, may evoke concomitant increase in DAG production by PAP2.

This finding regarding DAGK activation by insulin, which seems to be related with the DAG produced by phospholipases activated pathways, could thus be interpreted as a PA "recovery" signal mechanism.

Interestingly, this "recovery" mechanism induced by insulin is also reinforced by a direct DAGK activation mechanism exerted by the hormone. Insulin induced a sig-

nificant DAGK stimulation in the presence of exogenous DAG, which exhibits a complex fatty acid composition (Fig. 1). In addition, in Figure 2 it is shown clearly that insulin selectively stimulates a DAGK activity when 18:0-20:4 DAG was used as exogenous substrate. However, no insulin effect was observed in the enzyme activity when saturated DAGs were the substrate. It is well known that only DAGKε shows substrate selectivity (Luo et al., 2004). It could thus be hypothesized that the stimulatory insulin effect is exerted on this isoform. It is interesting to note that in the presence of R59022, which is not a good DAGKε inhibitor, insulin did not accumulate PA whereas a 5-fold increase in PIP_2 labeling occurred (data not shown), thus suggesting a fast PIP_2 resynthesis. In addition, and on the basis of the data showed in Figure 4 from pulse chase experiments (up to 5 min re-incubation) insulin plus neomycin accumulated PIP_2. It can be hypothesized that this insulin effect on DAGK could be related with PIP_2 resynthesis.

It has been shown that the CC PIP_2-signaling pathway is greatly affected in DAGKε −/− mutant mice (Rodriguez de Turco et al., 2001). It has been proposed that this enzyme contributes to modulating neuronal signaling pathways linked to synaptic activity and neuronal plasticity.

Taking into account our results regarding PLD-PAP2 pathway inhibitors that strongly blocked insulin effect, the generation of more saturated DAGs also occurs. It could be suggested that more than DAGKε are activated by insulin.

Our findings regarding the insulin effects on the stimulation of PA synthesis through the phosphorylation of DAG from a PIP_2-PLC and PLD-PAP2 activated pathways, and DAGK activation independently of the increased DAG production, show that insulin clearly promotes an overall PA "recovery."

It is still not possible to ascertain whether or not this insulin-stimulated PA generation is a signaling event or an efficient DAG inactivation mechanism by itself. It has been proposed that 18:0-20:4 DAG and PLD-PC derived PA are intracellular signalling molecules that often operate in parallel when receptors regulate cell function, and that other DAGs and PAs have no signalling functions (Hodgkin et al., 1998). However, recent studies have shown that PA formed by DAGKs may also have signaling roles (Luo et al., 2004).

A role for PLD1 in neurotransmitter release has been proposed (Humeau et al., 2001). By monitoring acetylcholine release from identified cholinergic neurons it was found that PLD1 plays a major role in neurotransmission by controlling the number of functional presynaptic release sites. Researchers explain PLD1 regulated fusogenic state by two possible mechanisms that involve the presence of PA rich domains, namely, 1) by recruitment or activation of a protein essential for fusion, 2) by an increased membrane curvature that promotes the mixing of vesicle and plasma membrane.

Insulin synthesis and IRs in the brain have been associated mainly to memory and knowledge (Zhao and Alkon, 2001). IRs are located at the synapse where they regulate neurotransmitter release and receptor recruitment, indicating a potential involvement of insulin in synaptic plasticity (Abbott et al., 1999). Evidence suggests that insulin has various functions in the CNS apart from its glucose uptake activity, such as increasing the expression of postsynaptic GABA A receptors (Wan et al., 1997), axon guidance during the development of the visual system (Song et al., 2003), and reducing both intracellular amyloid β and the level of τ phosphorylation in Alzheimer's disease (Hong and Lee, 1997; Gasparini et al., 2001). In addition, and similarly to other known neurotrophic factors, insulin may inhibit neuronal apoptosis (Ryu et al., 1999; Barber et al., 2001). Strong necrosis inhibition by insulin pre-treatment in a hypoxia-reperfusion stress model in culture cortical neurons has been reported recently (Hanabe et al., 2005). However, the molecular mechanism by which insulin exerts these beneficial effects has not yet been explained.

Our finding regarding the insulin-activated PA recovery mechanism that promotes not only the remotion of DAG generated by PLD-PAP2 but also DAG from PIP_2-PLC, seems to be the initial step in the study of neural action of this hormone. Our finding about the selective 18:0-20:4 DAG remotion and PA stimulated synthesis (with exogenous DAG) that was strongly activated by insulin, implies that other regulatory mechanism than that of the increase in DAG content by phospholipase activation is present in nerve terminal endings.

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